β, β-CAROTENE 15,15' ENZYMES, NUCLEIC ACID SEQUENCES CODING THEREFOR AND THEIR USE

FIELD OF THE INVENTION

[0001] The present invention concerns the cloning of β,β-carotene 15,15' enzyme (EC 1.13.11.21), the enzyme responsible for the cleavage of β-carotene leading to vitamin A. The term vitamin A as defined in the present invention comprises a class of compounds including retinal, retinol, 3-dehydroretinol, retinoic acid, the isomers from these compounds as well as retinylesters. Proteins having β,β-carotene 15,15' enzyme activity and nucleic acid sequences coding therefore can be used in different fields including but not limited to diagnostics, the technical production of vitamin A, the generation of transgenic plants in order to produce vitamin A in fruits and vegetables, or gene therapy.

SUMMARY OF THE INVENTION

[0002] In one embodiment of the present invention, a polypeptide having β,β-carotene 15,15' enzyme activity is provided. This polypeptide includes SEQ ID NO: 1 or a polypeptide having β,β-carotene 15,15' enzyme activity and being at least 60% homologous to SEQ ID NO: 1 as determined by the Wisconsin Sequence Analysis Package GCG, Version 9.1 (1997).

[0003] The present invention also includes a nucleic acid sequence encoding the polypeptide defined above, such as for example, SEQ ID NO: 2 or a fragment thereof.

[0004] Another embodiment of the invention is a primer for amplifying a gene coding for a protein having β,β-carotene 15,15' enzyme activity which includes a nucleic acid sequence as defined above.

[0005] A probe is also provided for detecting a gene coding for a protein having β,β-carotene 15,15' enzyme activity. This probe includes a nucleic acid sequence as defined above.

[0006] A test kit is also provided for amplifying and/or detecting a gene or a fragment thereof coding for β,β-carotene 15,15' enzyme. The test kit includes at least one primer as defined above. The test kit may also include at least one probe as defined above alone, or in combination with at least one primer according to the present invention.

[0007] Another embodiment of the invention is an antibody which specifically reacts with a polypeptide as defined above.

[0008] An immunoassay is also provided for the detection and/or quantification of β,β-carotene 15,15' enzyme. This immunoassay includes at least one antibody as set forth above.

[0009] A process is also provided for the production of vitamin A. This process includes enzymatically cleaving β-carotene by a polypeptide as described above.

[0010] Another embodiment is a method for introducing a β,β-carotene 15,15' enzyme cDNA into a host cell. This method includes inserting a cDNA coding for a polypeptide as described above into a vector suitable for the host cell and introducing the vector into the host cell.

[0011] A host cell is also provided. This host cell may be obtained by the method set forth above. The host cell includes a β,β-carotene 15,15' enzyme cDNA obtained from another species.

[0012] Another embodiment of the invention is a polynucleotide which encodes β,β-carotene 15,15' enzyme and includes the sequence of SEQ ID NO: 2.

[0013] A vector is also provided which includes the sequence of SEQ ID NO: 2. A host cell is also provided which has been transformed with this vector.

[0014] The present invention also includes a polypeptide having β,β-carotene 15,15' enzyme activity, which polypeptide contains the amino acid sequences of SEQ ID Nos: 1 or 4.

[0015] A primer set is also provided for amplifying a polynucleotide encoding β,β-carotene 15,15' enzyme. This primer set includes SEQ ID NO: 8 as a 5' primer and a SEQ ID NO: 9 as a 3' primer. Another primer set for amplifying a polynucleotide encoding β,β-carotene 15,15' enzyme is also provided which includes a polyT/Not reverse primer and SEQ ID NO:10 as a forward primer.

[0016] The present invention also includes a kit for amplifying and/or detecting a polypeptide or fragment thereof encoding β,β-carotene 15,15' enzyme. This kit includes at least one primer selected from SEQ ID Nos: 8, 9, and 10.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figure 1 shows the result from the last step of purification of β,β-carotene 15,15' enzyme from the small intestine of chicken. The SDS-PAGE pattern and β,β-carotene 15,15' enzyme activity of individual fractions from the gel permeation chromatography run are shown. On the gel, the protein A marked by an arrow correlated best with the β,β-carotene 15,15' enzyme activity. It was therefore chosen for further amino acid sequence analysis. The abbreviations have the following meaning: Std.: molecular weight standard; conc.: concentrate loaded onto the gel permeation chromatography column.

[0018] Figure 2 shows schematically the transactivation assay in eukaryotic cells. cDNAs are transfected and expressed in MCF-7 cells. When incubated with β-carotene, a positive pool shows cleavage activity. The cleavage product retinal is further oxidized to retinoic acid (RA) which binds to the endogenous receptor. The receptor/ligand complex binds to the response element on the reporter plasmid and leads to an enhanced transcription of the luciferase gene. The luminescene signals are detected in a luciferase assay with a sensitive CCD camera.

[0019] Figure 3 shows the cDNA sequence (SEQ ID NO: 2) for β,β-carotene 15,15 enzyme which has a length of 3090 base pairs excluding the poly A tail. 132 base pairs are 5' nontranslating sequence, the coding sequence has 1578 base pairs and the 3' nontranslating sequence 1380 base pairs, respectively. A putative poly A signal is found at position 3073.

[0020] Figure 4 shows the derived amino acid sequence (sequence ID No. 1) of β,β-carotene 15,15' enzyme derived from chicken having 526 residues. The amino acid sequence is given in the one letter code.

[0021] Figure 5 shows a comparison of the β,β-carotene 15,15' enzyme amino acid sequence (SEQ ID NO: 4) with a protein having the designation RPE65 (SEQ ID NO: 5) which was found by a sequence comparison in EMBL Genbank as the protein having the highest homology to the β,β-carotene 15,15' enzyme of the present invention.

[0022] Figure 6 shows two fractions of β,β-carotene 15,15' enzyme eluted from a Co^{2+}-chelate column. In lanes 1 and 2 two different fractions were loaded and lane 3 is a low range molecular weight marker.

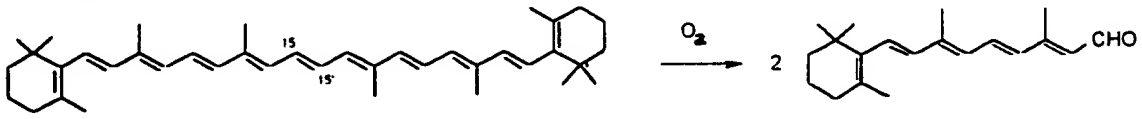
[0023] Figure 7 shows an HPLC analysis of an activity test of β,β-carotene 15,15' enzyme which was cloned and expressed in *E. coli*.

[0024] Figure 8 is a chromatogram demonstrating that the peak from Fig. 7 representing the only product of the enzymatic cleaving is retinal.

DETAILED DESCRIPTION OF THE INVENTION

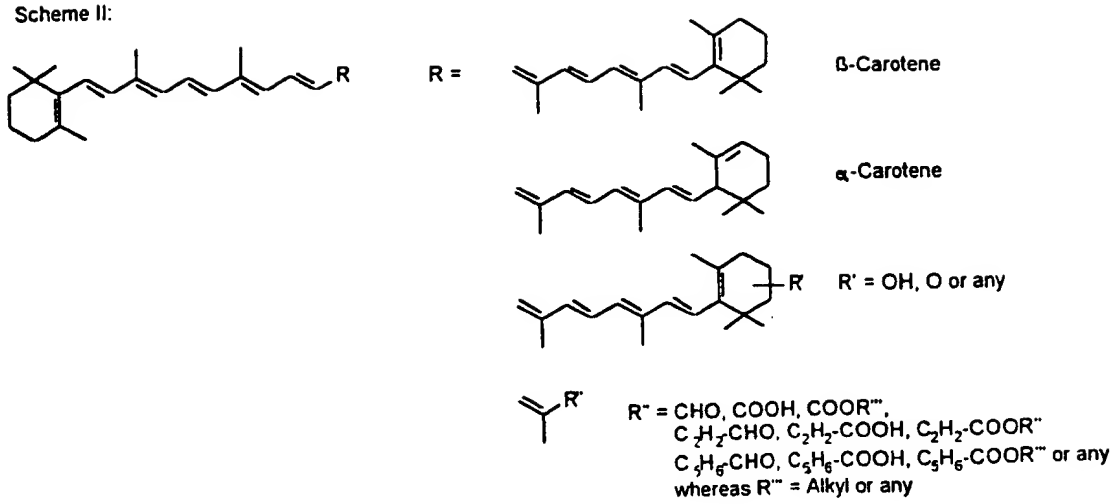
[0025] Vitamin A is essential for man and animal and is largely formed in most organisms from its precursor carotenoids which, by themselves, can only be formed in plants, in photosynthetic active microorganisms and some other microorganisms. Man and most animals (in particular herbivores and omnivores) are able to convert such carotenoids, also called provitamins A, enzymatically into vitamin A. The most important enzyme for this process is the β,β-carotene 15,15' enzyme (EC 1.13.11.21). The enzyme is located in the cytosol and forms retinal from β-carotene, as the principal substrate, in presence of oxygen according to scheme 1:

Scheme I:



[0026] The enzyme β,β-carotene 15,15' enzyme is characterized by generating 2 mols retinal from 1 mol of β-carotene by central cleavage. But the enzyme is also able to convert a wide range of carotenoids into vitamin A-active compounds, as shown in scheme II:

Scheme II:



[0027] Highest known enzymatic activity is found in the intestine of herbivores, especially in duodenum. In other tissues like liver, lung, kidney and brain β,β-carotene 15,15' enzyme is also detectable. Starting in 1955, many attempts have been undertaken to purify and characterize the enzyme by biochemical methods (Goodman (1965 and 1966), Fidge (1969), Laksmanan (1972), Sharma (1977) and Devery & Milborrow (British Journal of Nutrition (1994) 72, p. 397-414). However, none of these attempts has been successful. Specific activities of 600 pmol retinal formed/mg protein per hour have not been surpassed.

[0028] In the course of the present invention it was possible to purify the chicken enzyme to such a degree that a partial amino acid sequence could be obtained. The enzyme was enriched 226-fold, yielding a specific activity of 2500 pmol/h/mg. On a polyacrylamide gel of fractions from the final gel filtration run 15 bands were visible after Coomassie blue staining. Two bands correlated with the enzymatic activity profile of the β,β-carotene 15, 15' enzyme. With the first protein Edman sequencing and with the second MS spectroscopy was performed. Tryptic ingel digestion and subsequent microbore RP-HPLC peptide mapping in combination with MALDI-TOF MS and automated Edman degradation of this latter protein revealed 2 peptides of 11 and 18 amino acids. From this sequence information degenerate PCR primers were designed and synthesized.

[0029] With a PCR protocol a 51 bp (base pair) fragment was amplified within the longer peptide. From this sequence a homologous primer was synthesized and used in a second RT-PCR (reverse transcriptase - PCR) to amplify a 597 bp fragment.

[0030] This cDNA fragment was radioactively labeled and used for the screening of two positive pools from a chicken expression library in order to isolate the full length cDNA coding for β,β-carotene 15,15' enzyme.

[0031] The positive pools were obtained from a cDNA library from chicken duodenum which was screened for β,β-carotene 15,15' enzyme activity in a cellular transactivation assay. By this strategy several positive cDNA pools were identified. By combining the two strategies the gene coding for β,β-carotene 15,15' enzyme could be successfully cloned.

[0032] It is an object of the present invention to provide a protein having the vitamin A producing activity of β,β-carotene 15,15' enzyme comprising an amino acid sequence which is identical or homologous to SEQ ID NO: 1 (shown in Fig. 4) whereby the degree of homology to SEQ ID NO: 1 is at least 60%.

[0033] With the sequence of β,β-carotene 15,15' enzyme isolated from chicken, corresponding proteins from different animals like swine, cow, goat, dog, rabbit, poultry, fish and humans can easily be obtained. Since the chicken sequence is known, suitable regions of the nucleic acid sequence can be selected as primers for a polymerase chain reaction with a suitable nucleic acid which allows an easy and rapid amplification of the gene coding for the protein.

[0034] The present invention includes therefore not only proteins having an amino acid sequence identical to the sequence given in SEQ ID NO: 1 but also such proteins which have an amino acid sequence homologous to the SEQ ID NO: 1. The degree of homology is, however, at least 60%, preferably 70%, more preferably 80%, such as for example, at least 90%. Homology as defined in the present invention means that when the amino acid

sequences of two proteins are aligned at least the given percentage is identical. The alignment of the amino acids is performed with the help of a suitable computer program which is commercially available, in particular, the Wisconsin Sequence Analysis Package GCG (Genetics Computer Group, University Research Park, Madison), Version 9.1, 1997. The remainder of the amino acids may be different. A homology of 90% for example means that 90% of the amino acids of the protein are identical compared with the amino acid sequence given in SEQ ID NO: 1 whereas 10% of the amino acids may be different. The proteins of the present invention have, however, the biological activity of β,β-carotene 15,15' enzyme which is explained above in more detail. In the present invention, this protein may be derived from other sources, for example, from other mammals, such as from humans.

[0035] As used herein, the terms "protein" and "polypeptide" are used interchangeably throughout. The terms "nucleic acid" and "polynucleotide" are likewise used interchangeably.

[0036] The term "nucleic acid" is intended to include, without limitation, DNA, RNA, cDNA, and mRNA. As used herein, the DNA may be genomic, synthetic, or semi-synthetic. Moreover, the nucleic acids of the present invention include single-stranded and double stranded molecules.

[0037] As used herein "derived from" means that the protein, polypeptide, and/or polynucleotide exists naturally in an organism, such as for example, a chicken. However, the polypeptides and polynucleotides of the present invention may be produced/obtained from any source. Thus, the present invention includes recombinant, synthetic and semi-synthetic proteins, polypeptides, and polynucleotides.

[0038] The compositions of the present invention are said to be "isolated," such as for example "isolated polypeptide," "isolated polynucleotide," etc. As used herein, the term

"isolated" is intended to mean that the polypeptide or polynucleotide is purified or, at least partially purified as described, for example, in Fig. 1.

[0039] Another aspect of the present invention concerns nucleic acid sequences coding for a protein having the biological activity of β,β-carotene 15,15' enzyme. A nucleic acid sequence coding for the enzyme derived from chicken is shown in SEQ ID NO: 2 (see Figure 3). The nucleic acid sequences of the present invention code for a protein of the present invention or a part thereof. Shorter nucleotide sequences suitable for PCR have a length of at least 20 bases, preferably at least 25 bases and most preferred at least 30 bases.

[0040] The nucleic acid sequences of the present invention can be used as primers for the specific amplification of a gene or part thereof coding for β,β-carotene 15,15' enzyme. Primers can also be used for the specific amplification of 5' nontranslating or 3' nontranslating sequences of the cDNA described above. The nucleic acid sequences of β,β-carotene 15,15' enzyme cDNA can be used as a probe for the detection of the coding as well as for the noncoding regions or parts thereof. The nucleic acid sequences of the present invention can be used as antisense RNA probes for *in situ* hybridization.

[0041] It is especially preferred to use primers and probes having a part of the sequence given in SEQ ID NO: 2 as primers and/or probes in test kits which can be used for the amplification and/or detection of genes/mRNAs coding for β,β-carotene 15,15' enzyme by the polymerase chain reaction (PCR). The selection of suitable parts of the nucleic acid sequence can be performed by the person skilled in the art without difficulties. A nucleic acid sequence used as a primer or probe is usually selected from a region which is highly conserved within the protein. Conserved means that the nucleic acid sequences of such regions of proteins obtained from different species are very similar.

[0042] On the other hand the preferred nucleic acid sequence should not be present in other nucleic acid sequences which do not code for β,β-carotene 15,15' enzyme, because this might lead to false positive results. By aligning several sequences derived from

different species such regions can easily be determined. Although the nucleic acid can be a ribonucleic acid it is more preferred to have deoxyribonucleic acid sequences.

[0043] One preferred use in diagnostics is the detection of the presence of β,β-carotene 15,15' enzyme in patients. There is variability in β-carotene cleavage potential among the human population. Humans with low β,β-carotene 15,15' enzyme levels (with e.g. mutations or polymorphisms in the gene for β,β-carotene 15,15' enzyme) could be identified and selected for vitamin A supplementation.

[0044] A diagnostic kit based on PCR can be designed to detect frequent mutations in the enzyme gene. Another diagnostic option is quantification of mRNA by RT-PCR. With this diagnostic tool differences in expression of β,β-carotene 15,15' enzyme in various tissues and in different species can be found.

[0045] Since the protein has been expressed and a method for purifying the protein is described in detail in the examples the person skilled in the art can use the protein or peptides derived from the amino acid sequences in order to generate antibodies which specifically react with the protein. It is either possible to produce polyclonal antibodies by immunizing laboratory animals, like rabbits, sheep or goats preferably with an adjuvant or monoclonal antibodies by the well-known technique described by Köhler and Milstein (European Journal of Immunology, 1976, 6 (7), p. 511-519). The antibodies should specifically react with β,β-carotene 15,15' enzyme in order to avoid an unspecific crossreaction. This means that the antibodies of the present invention should preferably react with an epitope which is present only on a protein of the present invention.

[0046] Such antibodies can be preferably used in immunoassays for the detection and/or quantification of β,β-carotene 15,15' enzyme in a test fluid. The test fluid may be a liquid, like serum, obtained from a patient. There are several types of immunoassays which are well-known to the person skilled in the art. Very frequently one antibody, preferably a monoclonal antibody is fixed to a solid phase. This antibody is then brought into contact

with the fluid containing the β,β-carotene 15,15' enzyme and after washing it is further reacted with a second monoclonal antibody which binds to another epitope of the enzyme. The second antibody is usually labeled and shows the presence of the sandwich consisting of the antigen and two different antibodies.

[0047] The antibodies can also be used in laboratory methods like Western blots or immuno-precipitations. Preferably such antibodies can be used in immunohistochemistry to detect epitopes of β,β-carotene 15,15' enzyme in embedded or fixed tissues or cells of any species of interest.

[0048] In a further embodiment of the present invention the β,β-carotene 15,15' enzyme is used for the production of vitamin A whereby the enzyme cleaves enzymatically β-carotene into two molecules of retinal which will subsequently be reduced by retinol dehydrogenase to vitamin A. The β,β-carotene 15,15' enzyme can be used to enzymatically convert β-carotene which may be obtained from plant sources. A preferred source of β-carotene is the alga *Dunaliella bardawil* which has a high endogenous level of β-carotene. Suitable algae can be grown conveniently and β-carotene can be purified therefrom at rather low cost. The carotene can be conveniently cleaved enzymatically by using a protein of the present invention. The carotene enzyme can preferably be immobilized in order to provide a continuous process.

[0049] Another aspect of the present invention concerns the introduction of the gene coding for a protein having β,β-carotene 15,15' enzyme activity into a suitable host cell. The first step in such a method is usually to insert the cDNA into a suitable vector. The vector must fit with the host cell into which the gene should be introduced. There are specific vectors available for bacteria, yeasts, plant cells, insect cells or mammalian cells. Preferably the gene is combined with genetic structures which provide the required genetic regulation like promotors, enhancers, ribosomal binding sites etc.

[0050] Systems for the expression of genes encoding carotenoid biosynthetic enzymes in procaryotes, especially in *E. coli* or *Bacillus subtilis* or *Flavobacter* and eucaryotes, e.g. fungi are known in the art and described e.g. in EP Publication Nos. 747 483 or EP 872 554.

[0051] The vector having the gene and the other required genetic structures is then introduced into suitable host cells by well-known methods like transformation, transfection, electroporation or microprojectile bombardment. Depending on the host cell it may be preferred to stably integrate the gene coding for a protein of the present invention into the genome of the host cell. The cells obtained by such methods can then be further propagated and if the cell is a plant cell it is possible to generate therefrom transgenic plants.

[0052] In one embodiment of the present invention the host cells are plant cells and tomato cells are especially preferred. The technology to produce transgenic tomatoes is well-established and the tomato contains sufficient β-carotene in order to come up with a reasonable vitamin A level after introduction of carotene enzyme into the tomato plant. In green pepper, melon or especially carrot the endogenous level of β-carotene is even higher and therefore also these plants are especially preferred.

[0053] Another preferred embodiment of the present invention concerns algae. Halotolerant algae may contain high levels of β-carotene. A transfection of such algae with an expression vector comprising the β,β-carotene 15,15' enzyme cDNA leads to a high intracellular vitamin A level which can easily be recovered from such algae by simple purification steps.

[0054] In another aspect of the present invention a gene coding for a protein of the present invention can be introduced into mammalian cells and especially into human cells. It is for example possible to insert the gene coding for a β,β-carotene 15,15' enzyme into suitable cells, for example peripheral blood stem cells. Such cells which contain the gene

for β,β-carotene 15,15' enzyme may be administered to people having mutations or deletions in the β,β-carotene 15,15' enzyme gene. Such mutations and deletions, respectively, may have the effect that such patients are not able to cleave β-carotene enzymatically. Therefore, such patients always have a low vitamin A level and thus suffer from various developmental and ophthalmological problems. The administration of suitably transfected cells expressing the β,β-carotene 15,15' enzyme to such patients by way of somatic gene therapy is a way to improve their situation.

[0055] The following examples are provided to further illustrate methods of preparation of the enzyme of the present invention, as well as certain physical properties and uses thereof. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

EXAMPLE 1

Assay of β,β-carotene 15,15' enzyme activity

[0056] For the tests the following solutions were prepared:

[0057] a. Solution 1 (mixed micelle solution): Glycocholic acid (1.16 g) was dissolved in 5 ml H_2O under stirring and by drop-wise addition of 5 N NaOH. After the pH was adjusted to 6.8-7.2 with acetic acid and the volume increased to 10 ml with H_2O, 80 mg of asolectin (Fluka) were added and dissolved under stirring.

[0058] b. Solution 2 (substrate solution): 500 µl of an α-tocopherol solution (10 mg/ml in hexane) and 235 µg of a β-carotene solution (80 µg/ml of pure all-E-β-carotene in benzene) were mixed in a glass vial, protected from light and the solvents evaporated under a gentle nitrogen stream. 1 ml of solution 1 was added under vortexing and eventually a few short ultrasonic bursts until a clear solution occurred.

[0059] c. Solution 3 (homogenization buffer): 100 mM KH_2PO_4 adjusted with 5 N KOH to pH 7.8 and containing 4 mM $MgCl_2$, 6 H_2O and 30 mM nicotinamide.

[0060] d. Solution 4 (GSH solution): 60 mg/ml reduced glutathione dissolved in solution 3.

[0061] e. Solution 5 (standard solution): 10 µg/ml vitamin A acetate in hexane/chloroform 9:1.

Activity Assay

[0062] 2 ml of the enzyme preparation (approximately 4 mg protein, assayed by BCA protein assay, Pierce Chemicals) were placed in a light-protected glass vial in a shaking water bath (30 minutes, 37 °C). 0.2 ml of solution 4 was added and the reaction was started after 2 minutes of temperature equilibration by addition of 50 µl of solution 2. After 3 hours, the reaction was stopped by placing the vials on ice and subsequent addition of 1 ml acetonitrile followed by 5 ml chloroform. The vials were vortexed 3 times for 7 seconds and phase separation was obtained by centrifugation for 5 minutes at 5000 g. Extraction was repeated twice with 0.6 ml chloroform. The combined chloroform phases were evaporated and resolubilized in 200 µl solution 5 under short sonication. Insoluble material was removed by filtration through 0.45 µm filters. An aliquot of 20 µl was separated by HPLC on a reversed phase C_{18} column (Lichrospher 100, 5 µm, 12.5 cm x 4.6 mm, Bischoff Chromatography, Leonberg, Germany; 1 ml/min, column temperature 25°C) with a discontinuous, optimized gradient of acetonitrile/tetrahydrofuran/(1% ammonium acetate in H_2O) from 50:20:30 (eluent A) to 50:44:6 (eluent B). These conditions allow complete separation of β,β-carotene and retinal as well as apo-β-carotenals and retinoic acids. Calibration curves were made for both β,β-carotene and retinal in the concentration ranges 2-40 ng/µl and 1-10 ng/µl, respectively, and were correlated to the value of vitamin A acetate which served as an internal standard. Enzymatic activity was expressed as the amount of retinal liberated in the activity assay during 3 hours of incubation at 37 °C (100% = 17.6 nmol).

EXAMPLE 2

Purification of β,β-carotene 15,15' enzyme

[0063] Purification was done as rapidly as possible, and all buffers and equipment were cooled to 4 °C.

[0064] Solution 6 (protease inhibitor-containing homogenization buffer): 125 mM benzamidine·HCl, 250 mM 6-aminocaproic acid and 125 µM soybean trypsin inhibitor were dissolved in H_2O by sonication. A 4 ml-aliquot of this solution was mixed with 100 ml of solution 3.

[0065] Solution 7: 10 mM KH_2PO_4, 1 mM reduced glutathione, pH 7.8.

[0066] Solution 8 (eluent A, phenyl-Sepharose chromatography): 10 mM KH_2PO_4, 1 mM reduced glutathione, 0.5 M $(NH_4)_2SO_4$, pH 7.8.

[0067] Solution 9 (eluent B, phenyl-Sepharose chromatography): 10 mM KH_2PO_4, 1 mM reduced glutathione, 10% glycerol, pH 7.8.

[0068] Solution 10 (eluent B, Poros HQ chromatography): 10 mM KH_2PO_4, 1 mM reduced glutathione, 0.5 M NaCl, 10% glycerol, pH 7.8.

[0069] Solution 11 (elution buffer for gel permeation chromatography): 50 mM KH_2PO_4, 1 mM reduced glutathione, 150 mM NaCl, 10% glycerol, pH 7.8.

[0070] Laying hens at an age of 20-24 weeks (strain Lohmann LSL, Hatchery Wuethrich, CH-3123 Belp, Switzerland) were kept on a pigment-free chicken diet (Kliba 3179, Kliba, CH-4303 Kaiseraugst, Switzerland). The animals were killed by decapitation and the first 20 cm of the duodenal loop was removed, separated from pancreas and rinsed

with 40 mL each of 0.9% NaCl solution. The intestines (duodenal loops) were immediately frozen in dry ice and stored at -80°C until use.

[0071] Ten intestines (duodenal loops) were thawed on ice in approximately 2 hours and opened length-wise in an ice cooled Petri dish. The mucosa was scraped off with a slide, weighed and homogenized in a Teflon-glass Potter-Elvehjem homogenizer in 4 volumes of solution 6 with six strokes. Upon centrifugation at 62000 g for 1 hour, the clear supernatant was divided into 32 aliquots of 15 ml each. From these preparations an ammonium sulphate fractionation was made. The precipitate obtained from the 20-45% step was centrifugated at 5000 g for 10 minutes and the pellet was stored at -80°C for further use.

[0072] Ten aliquots of the $(NH_4)_2SO_4$ pellet were dissolved in 150 ml of solution 7, sterile-filtered and loaded on a HiLoad 26/10 phenyl-Sepharose High Performance column (column volume 53 ml; Pharmacia, Uppsala, Sweden) and equilibrated with solution 8. Proteins were eluted at a flow rate of 8 ml/minute with a steep gradient over 1 column volume (CV) from solution 8 to solution 9. β,β-Carotene 15,15' enzyme eluted at a conductivity of < 15 mS/cm, but only fractions with a conductivity of < 1 mS/cm were pooled and directly loaded onto a 30 ml Blue Sepharose 6 Fast Flow column (Pharmacia) equilibrated with solution 9. β,β-Carotene 15,15' enzyme activity eluted (at a flow rate of 8 ml/min) in the break-through fractions which were (again) directly loaded onto a 20 ml Poros HQ/M anion exchange chromatography column (PerSeptive Biosystems, Framingham, MA, USA) equilibrated with solution 9. β,β-Carotene 15,15' enzyme was eluted at a flow rate of 15 ml/minute with a linear gradient over 18 CV from solution 9 to solution 10. Activity was detected in the gradient in a conductivity range of 10-20 mS/cm. The pooled fractions (70 ml) were concentrated to ~1.3 ml with Ultrafree-15 filter units (MW cut-off 50,000; Millipore, Bedford, MA, USA). An aliquot of the concentrate (500 µl) was loaded onto a Superdex 200 HR 10/30 gel filtration column (CV 24 ml; Pharmacia) and eluted at a flow rate of 0.5 ml/minute with solution 11. Aliquots of each

fraction were used for activity assays (see example 1) and, upon concentration, for SDS-PAGE (with MOPS running buffer) on 10% NuPAGE gels (Novex, San Diego, CA, USA).

[0073] The results of this experiment are shown in Fig. 1 and Table 1.

TABLE 1

Purification step	Total protein (mg)	Total activity (nmol/h)	Yield (%)	Specific activity (pmol/(h·mg))	Purification factor
$(NH_4)_2SO_4$ pellet	779	8.61	100	11.0	–
phenyl-Sepharose	80.8	8.27	96.1	102	9.27
Blue Sepharose	16.0	8.86	103	554	50.1
Poros HQ	1.56	3.90	45.3	2500	226

Table 1: Summary table for the purification of β,β-carotene 15,15' enzyme starting with 10 aliquots of the $(NH_4)_2SO_4$ pellet (means of 3-4 measurements).

EXAMPLE 3

Amino acid sequence information for β,β-carotene 15,15' enzyme

[0074] For amino acid sequence analysis, fractions of the gel filtration run (as shown in Fig. 1) were separated on a 8-16% Tris/glycine gel (Novex), and the proteins transferred to an Immobilon P^{SQ} membrane (Millipore) and stained with amido black.

[0075] Since protein A proved to be N-terminally blocked, multiple aliquots of fraction 18 from the gel filtration run (see Fig. 1) were separated on a 10% Tris/glycine gel (Novex), and the gel was stained with Colloidal Coomassie Blue (Novex). The band corresponding to protein A was excised from the gel, and the protein digested in-gel with trypsin. The tryptic digest was separated by micro-bore reversed-phase HPLC on a 150 x 1.0 mm Vydac C_{18} column (Vydac, Hesperia, CA, USA). Peptides were eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid, and peptide containing fractions were

collected for further analysis. Two fractions were identified by MALDI-TOF-MS (Voyager Elite, PerSeptive Biosystems) to contain one single peptide each. N-terminal Edman degradation revealed the following sequences:

(1) Ala-Glu-Val-Gln-Gly-Gln-Leu-Pro (Seq. ID No. 3)
(2) Asn-Lys-Glu-Glu-His-Pro-Glu-Pro-Ile-Lys-Ala-Glu-Val-Gln-Gly-Gln-Leu-Pro (Seq. ID No. 6)

[0076] Note that the last 8 amino acids of peptide (2) correspond to peptide (1).

EXAMPLE 4

Cloning the full length cDNA for the β,β-carotene 15,15' enzyme

A) RNA isolation:

[0077] A 4 week old Vedette chicken was killed, the duodenum was removed, washed with sterile PBS and cut open with scissors. The mucosal layer was scraped off with a glass slide, weighed and homogenized immediately with a Polytron in 1 ml of Trizole reagent (Life Technologies) per 100 mg of tissue. Then the standard protocol from Life Technologies was followed. Poly A mRNA was isolated by the polyATtract mRNA Isolation kit from Promega Corporation, Madison.

B) PCR and RT-PCR:

[0078] In the peptide sequence NKEEHPEPIKAEVQGQLP (peptide 2 of Example 3) (Seq. ID No. 7) two degenerate primers were designed: In order to have a lower degeneracy the base Inosin was used in one and in two wobble positions, respectively.

[0079] 5' primer: 5'AAC AAR GAR GAS CAY CCI GA 3' (Seq. ID No. 8)
(20 mer with a degeneracy of 16x)

[0080] 3' primer: 5'SAG CTG ICC CTG IAC YTC SGC 3' (Seq. ID No. 9) (21 mer with a degeneracy of 8x)

[0081] R= A or G, S= C or G, Y= C or T

[0082] The oligos were synthesized on a Pharmacia Gene Assembler Plus using standard phosphoramidite chemistry. Deprotection was done with 1 ml conc. ammonium hydroxide solution (Applied Biosystems) and final desalting was performed with a NAP 10 column (Amersham Pharmacia Biotech).

[0083] For PCR 100 ng of chicken duodenal cDNA were taken as template and the following steps performed: 94°C 30''; 52°C 30''; 72°C 1' for 40 cycles. The resulting band of 51 bp was cut out from a 10% polyacrylamide gel, electroeluted on DEAE paper at 300 V for 1.5 hours, eluted from the DEAE paper once with 40 µl and twice with 30 µl 1.5 M NaCl, 5 mM Tris, 0.5 mM EDTA, precipitated with 2.5 volumes of ethanol 100% and 1 µg glycogen, washed with 0.5 ml of 80% ethanol, dried and dissolved in 20 µl TE (10 mM Tris, 1 mM EDTA).

[0084] The resulting fragment of 51 bp was cloned into pGEM-T Easy, a commercially available T/A cloning vector (Promega Corporation, Madison). The corresponding cDNA sequence was determined by automated fluorescent sequencing on a Vistra DNA Sequencer 725 (Amersham Pharmacia Biotech).

[0085] From the above DNA sequence a homologous forward primer was derived:

5' TCTGAATTCCGGAGCCCATAAAAGC 3' (primer dioxy12) (Seq. ID No. 10)

[0086] At the 5' end an EcoRI site (underlined sequence) was introduced; the following 17 nucleotides are homologous to the previously obtained enzyme sequence.

[0087] In a RT-PCR reaction a polyT/Not primer (commercially available from Invitrogen, San Diego) was used as reverse primer together with primer dioxy12.

[0088] One tube RT-PCR kit from Boehringer Mannheim was taken and the corresponding protocol followed:

mix 1:
18.3 µl H_2O
2.5 µl DTT (100 mM)
1.0 µl dNTPs (10 mM)
1.0 µl oligo dT/Not (0.2 µg/µl) (3' primer)
1.0 µl dioxy12 (5' primer) (20 µM)
0.2 µl RNAse inhibitor (40 U/µl)
1.0 µl chicken duodenal total RNA (2.2 µg/µl)

25.0 µl

mix 2:
14.0 µl H_2O
10.0 µl RT-PCR buffer 5x
1.0 µl enzyme mix (AMV RT, Taq and Pwo DNA Polymerase)

25.0 µl

[0089] The 2 mixes were combined and the PCR protocol started on a MJ Research PTC200 DNA Engine.

50°C 30''
94°C 2'

94°C 30''
57°C 30'' 10 cycles
68°C 45''

94°C 30''
62°C 30'' 25 cycles
68°C 45'' + 3'' /cycle

68°C 7'
4°C over night

[0090] With this RT-PCR protocol a band of 597 bp was amplified from chicken total duodenal RNA. The PCR band was isolated from a 1% agarose gel, cloned into pGEM-T Easy cloning vector and subsequently sequenced. The original peptide is present in the sequence as well as an open reading frame over the whole sequence of 597 bp.

C) Chicken cDNA-library:

[0091] From chicken duodenal polyA$^+$ RNA cDNA was made with the Copy Kit (Invitrogen, San Diego) using a modified Gubler-Hoffman procedure. The cDNA was size-selected (0.9-5.5 kb) and cloned into the eukaryotic expression vector pcDNA1.1/Amp (Invitrogen).

[0092] Electroporation into *E. coli* Top10 was done with a Bio-Rad Gene Pulser II system following the standard protocol. This resulted in a cDNA library of 480,000 individual clones. The library was split into 250 pools with 1500-2500 individual clones each. Each pool was amplified in 100 ml LB medium: Bacterial growth was stopped at OD 0.8-1.0 by adding chloramphenicol to a final concentration of 170 µg/ml. Incubation was continued over night in order to increase the DNA amount.

D) Activity screening of the chicken cDNA library:

[0093] 90 of the above pools were tested for activity in a transactivation assay based on the detection of retinoic acid which is produced in eukaryotic cells after β-carotene cleavage. The principle of the activity test is shown in Fig. 2.

[0094] 5 µg of DNA from each pool were transfected with 20 µg of lipofectin (Life Technologies) into a reporter cell line bearing a luciferase reporter plasmid with a RARE (retinoic acid response element) in front of the tk promoter (Herpes simplex thymidine

kinase promoter). Transfections were done for 7 hours under serum free conditions. After 7 hours the transfection mix was removed and RPMI medium with 10% charcoal treated FCS (fetal calf serum) was added. After 20 hours of incubation β-carotene (β-carotene 10% CWS, F. Hoffmann-La Roche Ltd.) or a placebo formulation were added to the culture medium to a final β-Carotene concentration of 5 μM. Incubation was continued for 18 hours. Then cells were washed with PBS, and luciferase expression was measured after substrate addition with a nitrogen cooled slow scan CCD camera (AstroCam Ltd.) Exposure time usually was 8 min. Analysis was done with the Image Pro Plus 3.0 software package (Media Cybernatic, Maryland). 3 pools were strongly positive,7 pools showed weaker, but detectable activity.

[0095] One of the positive pools was plated on a square agar plate. 2 filters (nylon membranes, Gene Screen, NEN Research Products, Boston) were processed and screened with the radioactively ((α^{32}P) dATP, Amersham) labeled 597 bp PCR-fragment. From 9500 colonies screened, 14 were double positive. From 36 colonies picked, 5 showed the same pattern after restriction site analysis. 2 clones were sequenced from the 5' end and the original 51 bp sequence was found. Subsequently the whole cDNA was sequenced and confirmed twice.

[0096] All molecular biological procedures were done according to Sambrook, Fritsch and Maniatis, Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), which is incorporated by reference as if recited in full herein, if not otherwise mentioned.

[0097] The obtained cDNA sequence is shown in Figure 3 and the amino acid sequence deduced therefrom in Figure 4.

[0098] Figure 4 shows the derived amino acid sequence having 526 residues.

EXAMPLE 5

Sequence comparison

[0099] By sequence comparison with the EMBL Genbank a high homology between the known protein RPE65 (Hamel et al., J.Biol.Chem. (1993) p. 15751-15757) and the β,β-carotene 15,15' enzyme was found. A homology of 55.5% on the amino acid level was found. The sequence alignment is shown in Figure 5.

EXAMPLE 6

Expression of the cDNA for β-β-carotene 15,15' enzyme in E. coli

[0100] With PCR the coding sequence of the β-β-carotene 15,15' enzyme cDNA was amplified and the resulting fragment of 1578 bp was cloned into the EcoRI/BamHI site of the prokaryotic expression vector pQE-12 (Qiagen). The vector contains an in frame hexa-His affinity tag at the C-terminus of the enzyme. In addition, this plasmid contains a regulated promoter with two lacI repressor binding sites.

[0101] The *E. coli* strain M15pREP4 was transformed with the expression plasmid. For expression 1 l LB medium, containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, was inoculated with 30 ml of an overnight culture. Growth was allowed until OD_{600} of 0.6-0.8 was reached. At this point the culture was induced with 1 mM IPTG (isopropyl-β-thiogalactoside) and growth continued for another 1.5-2 hours. Bacteria were harvested by centrifugation and the pellet was frozen at -80°C.

[0102] The pellet was thawed by stirring in 20 ml extraction buffer (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM Tris-HCl, 1 mg/ml Tween 40; pH 7.8) including 2.5 mg/ml dodecyl-β-D-maltoside. 1 ml protease inhibitor cocktail (5.9 mM benzamidine-HCl, 10 mM 6-amino-caproic acid, 5 µM soybean trypsin inhibitor) was added at the same time.

[0103] The bacterial cells were lysed by a 4 minute treatment with a Polytron (Kinematica AG, Switzerland) using a PT7 unit following a 4 minute sonication with a Branson Sonifier 250.

[0104] The lysate was spun at 12000 x g and the supernatant purified over a Co^{2+}-chelate column (Talon Superflow Resin, Clontech, Heidelberg, Germany). The protein was eluted with 15-20 ml of extraction buffer containing 150 mM imidazole.

[0105] The fractions were loaded on a 10% polyacrylamide gel and the protein containing fractions dialyzed against 150 mM tricine, 5 mM $FeSO_4$, 3 mg/ml reduced glutathione, 0.21 mg/ml sodium cholate. The gel is shown in Fig. 6. The samples were then assayed in the enzyme activity assay.

EXAMPLE 7

Expression of recombinant β-β-carotene 15,15' enzyme in the human duodenal cell line HuTu80

[0106] With PCR the coding sequence of the β-β-carotene 15,15' enzyme cDNA was amplified and the resulting fragment of 1578 bp was cloned into the BamHI/XhoI site of the plasmid $pSFV_2gen$. This vector is part of the Semliki Forest Virus expression system, which works highly efficiently in most mammalian cells.

[0107] The plasmid was used for *in vitro* synthesis of recombinant RNA, which was subsequently electroporated together with a helper virus into BHK cells (baby hamster kidney cells) for production of a high titer virus stock. With an aliquot of this stock the human duodenal cell line HuTu80 was infected.16-18 hours after infection the cells were harvested and the pellet frozen at -80°C. Either the whole cell pellet, the cytoplasmic fraction or the membrane fraction was used in an activity assay. β-β-carotene 15,15' enzyme activity was found in the whole cell extract and in cytosolic fractions, while in the membrane fraction no activity was detected.

EXAMPLE 8

β-β-carotene 15,15' enzyme activity of the recombinant protein expressed in *E. coli* and in human cells

[0108] After expression in *E. coli* and purification over a metal chelate column, the protein shows cleavage activity with β-carotene as substrate. Retinal was the only product detected by HPLC after incubation with β-carotene. No apocarotenals or other metabolites were found. This was proved by HPLC analysis as shown in Figs. 7 and 8.

[0109] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.